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Characterization of the Fertility of Male Aromatase Knockout Mice

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ABSTRACT: Previous studies employing the male aromatase knockout (ArKO) mouse have indicated that local expression of estrogens appears to be important for the progression of spermatogenesis. In the absence of estrogen biosynthesis round spermatids are observed to undergo apoptosis and thus fail to differentiate into mature, elongated spermatids. This lesion appears to arise between the ages of 18 weeks and 1 year. To ultimately determine if the disruption to spermatogenesis arises earlier than 18 weeks, we performed an intensive study to examine the fertility of younger male ArKO mice. This involved an analysis of their mating capacity together with an extensive stereological analysis, determination of the in vitro potential of mature sperm, and sexual behavior. ArKO and wild-type (w/t) males at 7 weeks of age were placed with w/t females for 7 weeks. At age 14 weeks, the males were killed and the testes removed. ArKO mice were observed to sire significantly fewer litters than the w/t mice; 5 out of the 10 sired no litters at all. Stereological analysis performed on the removed testes found a significant decrease in round spermatid numbers between w/t and ArKO mice at this age; however, there were no differences in all other germ cells and Sertoli cell numbers. When mature spermatozoa were analyzed, sperm from 15-week-old ArKO mice had a significant reduction in motility. This was further reduced by 1 year of age with a decrease in concentration. A preliminary examination of sexual behavior found that ArKO mice did not attempt to mount the females, in contrast to the w/t mice, which mounted consistently during the time period. In conclusion, we observed that ArKO mice have reduced fertility at age 14 weeks. This may be due in part to a disruption in spermatogenesis because the phenotype does appear to arise earlier than 18 weeks, possibly leading to abnormalities in the mature spermatozoa. Or, in part, this may be attributable to an impairment in the development of copulatory behavior, which is consistent with the available evidence that points to a crucial role for estrogens in the neural development and initiation of male sexual behavior.

Key words: Estrogen, spermatogenesis, sexual behavior.

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The role of estrogens in male reproduction, particularly testicular function, has only recently begun to be understood. This current influx of knowledge is due largely to gene targeting technology, which has created models designed to elucidate estrogen action. One of these models, developed in our laboratory, is the aromatase knockout (ArKO) mouse (Fisher et al, 1998). These ArKO mice lack a functional aromatase cytochrome P-450 enzyme, and thus are unable to catalyze the conversion of C19 steroids (androgens) to C18 steroids (estrogens). Our recent results (Robertson et al, 1999) suggest a role for estrogens in spermatogenesis. We observed that initially, spermatogenesis progresses normally, but disruptions appear to develop between 18 weeks and 1 year of age, although younger animals were not analyzed. This disruption is characterized by a significant decrease in the number of round and elongated spermatids, and appears to be due to specific lesions in early round spermatid maturation, leading to an increase in germ cell apoptosis. Studies have shown that aromatase is expressed in high levels at the site of disruption, in late pachytene spermatocytes and early round spermatids (Nitta et al, 1993). It is these germ cells that are known to express estrogen receptor β (ERβ) in the rat (Herbosa et al, 1996; Enmark et al, 1997; Saunders et al, 1998; van Pelt et al, 1999), however, information ascertained from mice that are homozygous for a deletion in ERβ (βERKO) show that they remain fertile (Ogawa et al, 1999). In contrast, nullifying ERα (αERKO), which does not appear to be expressed in the germ cells, has established that estrogen does play an important, albeit indirect role in fluid reabsorption at the site of the efferent ducts of the testis (Eddy et al, 1996; Hess et al, 1997; Lee et al, 2000).

In the male, spermatogenesis would be futile without the ability to copulate. Unlike many male reproductive structures, the development and maturation of which are dependant on androgen stimulation, the development of male-specific sexual behaviors requires these androgen precursors to be aromatized to estrogens (Meisel and

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Sachs, 1994). In fact, estrogen is synthesized locally in the sexually dimorphic neurons of the rodent medial preoptic area, the site implicated in the control of sexual behavior, in higher levels in the male than the female (for a review see Hutchison, 1997). This is demonstrated by the discrete localization and activity of the enzyme aromatase cytochrome P-450 in rat (Tobet et al, 1985; Lephart et al, 1992; Roselli and Resko 1993) mouse (Beyer et al, 1993; Karolczak et al, 1998), ram (Roselli et al, 1998), and quail brain (Balthazart et al, 1990b; Balthazart and Surlemont, 1990).

Estrogen biosynthesis is vital in the perinatal stage of development if masculine-type sexual behavior is to be exhibited in adulthood (Roselli and Resko 1993; Negri-Cesi et al, 1996; Hutchison et al, 1997). Treatment with an aromatase inhibitor (Balthazart et al, 1990a; Gonzalez and Leret 1992; Houtsmuller et al, 1994) or removing the ability to synthesize estrogens during this period (Honda et al, 1998) results in a significant reduction in copulatory behavior in adulthood, which is explicable in terms of preventing this testosterone conversion. However, estrogen synthesis is also required in adulthood for the continual display of sexual behavior in response to female stimuli. Castrating mature males or treating them with an aromatase inhibitor leads to a failure to mount in many species, including quail (Alexandre and Balthazart 1986; Watson and Adkins-Regan 1989; Balthazart et al, 1990a), mouse (Wallis and Luttge 1975; Clemens and Pomerantz 1982; Wee et al, 1988), rat (Clancy et al, 1995), ram (D'Occhio and Brooks, 1980), boar (Parrott and Booth, 1984), and ferret (Carroll et al, 1988). Treatment with either estrogens or aromatizable androgens, in the case of castration, restores this behavior.

To further investigate the disruption to spermatogenesis and to decipher if it arises earlier than 18 weeks of age, we characterized younger ArKO mice (14 weeks) for their mating potential, the progression of spermatogenesis, concentration and motility of mature spermatozoa, and sexual behavior. We observed that spermatogenesis does not progress normally in the 14-week-old animals, through disruptions in sperm development. We therefore suggest that the phenotype can arise earlier than 18 weeks and suggest that male ArKO mice may sire fewer litters due to disruptions in either spermatogenesis or mounting behavior.

Materials and Methods

Generation of ArKO Mice

The method used for targeted disruption of the aromatase *cyp19* gene has been described elsewhere (Fisher et al, 1998). Briefly, exon 9 was selected for disruption by insertion of the neomycin resistance gene, because the coding region between the *Eco*RV (bp 1047) and *Xho*I (bp 1210) sites is crucial for enzyme function (Graham-Lorence et al, 1995), and is highly conserved across species (Simpson et al, 1997).

Mouse Chow

Glen Forrest Stockfeeders (Glen Forrest, Western Australia) manufactured a gamma-irradiated mouse chow, which contained no soy products.

Animals

ArKO and wild-type (w/t) mice were housed in a 12-hour light/dark cycle in the Specific Pathogen Free facility at Monash Medical Centre Animal House, and were fed a soy-free mouse chow ad libitum. All experiments were approved by the Animal Experimentation Ethics Committee at Monash Medical Centre. Male w/t and ArKO mice at age 50 (± 1) days were obtained from the same colony. They were housed with either a known fertile heterozygote or w/t female, one breeding pair per cage, for 50 days (± 1), and the number of litters and pups were documented. Male mice were killed at the end of the breeding period.

Collection of Tissue and Stereology

The testes were dissected out, fixed, stained, and examined as previously reported (Robertson et al, 1999).

Sperm Data

W/t and ArKO mice at ages 15 weeks and 1 year were killed and epididymides removed. Spermatozoa were extracted as described (Lacham-Kaplan and Trounson, 1993), then analyzed for concentration, motility, and in vitro fertilization potential. Briefly, after removal of the cumulus mass, oocytes were fertilized with spermatozoa. If a spermatozoon was present under the zona pellucida these were then observed for a second polar body and 2 pronuclei to verify that fertilization was successful. These were then observed every 10–14 hours for development to the blastocyst stage.

Male Sexual Behavior

W/t (n = 5) and ArKO (n = 4) mice at ages 12–14 weeks from the breeding experiment were removed from their cages and observed for mounting behavior. Known fertile female w/t mice (from the ArKO colony) were ovariectomized and injected intraperitoneally with 10 μg estradiol 48 hours before the test, then with 500 μg progesterone 4–7 hours before test (Ogawa et al, 1998). All tests were performed during the dark phase. Male mice were placed in a viewing cage where they remained for 5 minutes to adjust to their new surroundings before the female was introduced. An infrared illuminator, using 10 infrared LEDs (light emitting diodes [MS-1570B]) mounted on a perspex strip (assembled by authors), was used to illuminate across the cage. This was attached to a MOD-BW miniature video camera (Go Video N266, Tandy Electronics, Perth, Australia) which was used to film the mice for 20 minutes. For each male the number of mounts, mount latency, and general behavior was recorded, and the tapes were viewed if further examination was required. If the female appeared to be unresponsive, she was replaced with another female in the viewing cage.

Table 1. *Fertility**

Animals		Number of Litters	Time to First Litter (Days)
w/t (n = 8)		1.75 ± 0.3	33.88 ± 3.5
ArKO			
(n = 10)	(5)*	1.20 ± 0.2†	28.20 ± 3.3
	(5)	0	...

* Male mice at age 50 (± 1) days were placed with known fertile females for 50 days. Five out of the 10 ArKO breeding pairs sired significantly less litters than w/t, the remaining pairs sired no litters. Results are shown as mean ± SEM.
† $P < .05$.

Statistical Analysis

All data were analyzed with the F-test to determine normality, and the appropriate t-test was applied at the level of 5% ($P < .05$). Data are expressed as means ± SEM.

Results

Fertility

W/t males (n = 8) at the age of 14 weeks sired an average of 1.75 (± 0.3) litters in the 50 days (Table 1). However, the ArKO mice (n = 10) had reduced numbers of litters. Five out of the 10 sired 1.2 (± 0.2) litters, significantly less than the w/t mice, whereas the remaining 5 sired no litters. There were no differences in the number of days until the first litter between the w/t and ArKO mice, nor in the number of pups per litter (data not shown).

Germ Cell and Sertoli Cell Numbers

Quantitation of cell numbers with the optical dissector technique revealed that the numbers of spermatogonia, spermatocytes, and elongated spermatids did not differ between the 14-week-old w/t (n = 12) and ArKO (n = 10) mice (millions of cells per testis: spermatogonia, 4.21 ± 0.3 vs 4.14 ± 0.4; spermatocytes, 25.84 ± 1.5 vs 26.92 ± 2.3; and elongated spermatids, 41.94 ± 2.5 vs 37.04 ± 3.8). However, there was a significant decrease in round spermatids (42.86 ± 3.0 vs 38.40 ± 1.09 million per testis; $P = .05$). Further analysis noted that one ArKO mouse, which presented with the disrupted phenotype, exhibited very few round and elongated spermatids, whereas 2 others had obvious disruptions to the spermatogenic process, from missing an entire class of germ cell (either spermatocytes or round spermatids), to spermiogenic arrest. Sertoli cell numbers were unchanged between the w/t and ArKO mice (3.62 ± 0.1 vs 3.77 ± 0.4 million per testis).

Quantitative Histomorphometry

Using stereological methods, no changes were observed in the volumes of seminiferous epithelium, interstitium, or lumen between the normal-appearing ArKO and w/t mice (data not shown). However, the 14-week-old animal that presented with severe disruptions to spermatogenesis showed a significant increase in testis interstitial volume. Testicular weight did not differ between the 2 groups.

Table 2. *Sperm data**

	Sperm Concentration (number/mL) ($\times 10^6$)	Sperm Motility (%)
w/t 15 wk	59.3 ± 19.1 (n = 4)	81.0 ± 4.1 (n = 4)
ArKO 15 wk	65.1 ± 7.5 (n = 7)	67.5 ± 1.7† (n = 6)
w/t 1 y	41.6 ± 10.6 (n = 5)	72.0 ± 2.0 (n = 5)
ArKO 1 y	10.75 ± 5.6† (n = 5)	24.0 ± 13.4† (n = 5)

* At 15 weeks, ArKO sperm concentration is unaffected, however, they have decreased motility. By 1 year of age ArKO sperm is present in a much lower concentration and the motility is severely compromised. Results are shown as mean ± SEM.
† $P < .05$.

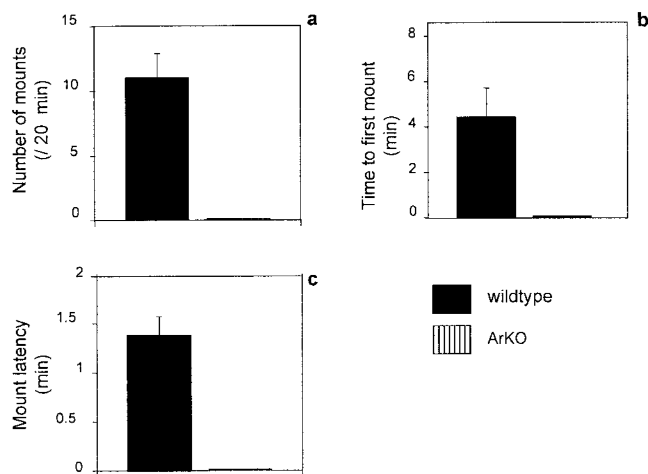
Sperm Viability

To determine whether the reduction in fertility of the ArKO animals was due to the viability of the mature spermatozoa being compromised, we performed an in vitro analysis of the sperm (see Table 2). The 15-week-old ArKO males had normal sperm concentrations but decreased motility. However, sperm from 6 out of 7 ArKO mice were able to fertilize oocytes in vitro (data not shown). This is in contrast to 1-year-old ArKO males whose sperm were unable to fertilize in vitro. This appears to be associated with a significant decrease in sperm concentration and motility (Table 2).

Sexual Behavior

Sexual behavior appeared normal in the w/t male animals at ages 12–14 weeks, in that they approached the female, repeatedly sniffed the anogenital region, and then mounted an average of 11 (± 1.9) times in 20 minutes (Figure). The first mount occurred within only 4.41 (± 1.3) minutes and each subsequent mount occurred regularly, with a latency period of 1.3 (± 0.2) minutes. In contrast, the ArKO mice demonstrated impaired mounting behavior. When placed with the females, males immediately interacted with the female and appeared to sniff her genital area, similar to the w/t males. However, this soon ceased and the male withdrew. No attempt to mount was initiated from any of the 4 males.

We also studied 1-year-old males and found that no ArKO males (n = 4) mounted in the 20-minute time period. However, there was also an impairment in the ability



Behavioral data. The w/t males at age 12–14 weeks mounted (**a**) 11 ± 1.9 times within (**b**) 4.41 (± 1.3) minutes until the first mount and (**c**) with a 1.37-minute (± 0.2-minute) latency between mounts. In comparison, no ArKO males were observed to mount. Results are shown as mean ± SEM.

of the 1-year-old w/t males (n = 4) to mount, with only 2 mounting (data not shown).

In conclusion, the 4 males tested did not attempt to mount females. Out of these 4, only 1 sired no litters, whereas the other 3 appeared to be fertile. None of these 4 presented with the phenotype. However, the animal with the severe disruptions did not sire any litters, whereas the other 2 did produce pups. Therefore, the fertility of the male ArKO mice may be in part attributable to abnormalities in either mounting or spermatogenesis, but most probably not both.

Discussion

We have previously established that aromatase appears to have a specific role in spermatogenesis (Robertson et al, 1999). Without estrogen synthesis, germ cells are observed to undergo apoptosis and, as a consequence, fail to form mature, round spermatids. However, this lesion appeared to develop between the ages of 18 weeks and 1 year. To further analyze the late onset of this spermatogenic phenotype, we undertook the same intense stereological analysis on younger, 14-week-old mice, in conjunction with an extended analysis of their fertility, including mating capacity, in vitro spermatozoa capabilities, and sexual behavior. We observed that male ArKO mice at age 14 weeks sired significantly fewer litters than their w/t colony mates; in fact, half of the ArKO males sired no litters at all. Stereological analysis of the volume of the testicular compartments (lumen, interstitium, and seminiferous epithelium) revealed no morphological differences between genotypes. Further examination showed no difference in the mean number of spermatogonia, spermatocytes, elongated spermatids, as well as Sertoli cells. However, there was a significant decline in the number of round spermatids. This led us to the conclusion that spermatogenesis was not progressing normally at 14 weeks in the 11 ArKO animals. Further histological analysis identified 3 animals that had clearly begun to present with a disrupted phenotype. One animal exhibited severe disruptions with no elongates present; therefore, passage through spermatogenesis was impaired at the early round spermatid/late spermatocyte stage, as is the case with older ArKO males. Also, the interstitial volume was increased in this testis, most likely due to Leydig cell hypertrophy. Two others exhibited various spermatogenic disruptions. Therefore, the decrease in fertility observed with the ArKO males may be attributable to a defect in spermatogenesis.

The one difference, however, between this study and our previous one (Robertson et al, 1999) is that these animals were fed a diet containing no soy. There have been numerous studies concerned with the role of exogenous estrogens on the male reproductive tract (Sharpe and Skakkebaek, 1993; Santti et al, 1998). The presence of phytoestrogens in the diet was also reported to have significant effects on the reproductive potential of adult males (Whitten and Naftolin, 1998). Because it appears that the onset of the ArKO disruption can occur over a broad range of ages when influenced by the presence of estrogenic substances in the diet, the role of phytoestrogens are currently being further examined.

Mature spermatozoa were analyzed for their ability to fertilize oocytes in vitro and to instigate development past the zygote stage. There was no difference between 15-week-old w/t and ArKO animals in this respect. However, sperm from 1-year-old animals was compromised and unable to fertilize oocytes (data not shown), which is consistent with the spermatogenic phenotype of these animals (Robertson et al, 1999) and with the decreased sperm concentration and motility (Table 2). This finding differs from those observed with the αERKO mice in which the sperm are incapable of fertilization at this young age. However, similar to the αERKO males, the sperm of our mice were less motile at 15 weeks of age (Eddy et al, 1996). As the ArKO mice age, the ability to fertilize declines, which may correspond with the late-onset disruptions observed in spermatogenesis (Robertson et al, 1999).

One explanation for the significant decrease in fertility we observed with the younger ArKO males could be an impairment in copulatory behavior. In fact, our preliminary examination of the sexual behavior of these mice, and that of Honda et al (1998), found that they appeared to have an inability to initiate mounting. This compared with w/t males, which mated readily with sexually receptive females. Considering past literature on male sexual

behavior, which emphasizes the importance of estrogen synthesis locally in the medial preoptic area during development, this inability to display masculine-type sexual behavior is not unexpected (Meisel and Sachs, 1994). However, a more specific study is required to fully determine which aspect of sexual behavior may be disrupted in the ArKO mice. When observing their behavior, following being placed with a hormonally primed female, we found that they initially approached the receptive females; however, compared with the w/t males, they appeared more hesitant and may have been curious rather than sexually motivated. The mice then undertook what appeared to be a nonsexual partnership.

The importance of aromatase in sexual behavior is observed when aromatase inhibitors are used. Following administration either shortly after birth or in adulthood, copulatory behavior is inhibited in quails, rats, mice, and ferrets (Clemens and Pomerantz 1982; Carroll et al, 1988; Balthazart et al, 1990a; Gonzalez and Leret, 1992; Clancy et al, 1995). However, this importance of aromatase may have species specificity. A study employing adult Syrian hamsters showed that administration of aromatase inhibitors systemically for 5 to 8 weeks failed to have any effect on their mounting behavior (Cooper et al, 2000).

In comparison with the ArKO mice, male sexual behavior is only partially disrupted in knockout mice that lack the gene for either ERα (αERKO) or ERβ (βERKO) individually (Lubahn et al, 1993; Krege et al, 1998). Two further studies indicated that although αERKO mice are infertile and rarely ejaculate, they showed either normal levels of mounts and just reduced levels of intromissions (Ogawa et al, 1997), or a reduction in both mounting and intromissions (Wersinger et al, 1997). Administration of testosterone or dopamine restored mounts and intromissions in gonadectomized αERKO mice; however, ejaculation was not restored, suggesting that it is regulated through pathways other than ERα (Ogawa et al, 1998; Wersinger and Rissman, 2000).

In contrast, all 3 components of sexual behavior were present and robust in the βERKO male (Ogawa et al, 1999). On the other hand, the double knockout (αβ-ERKO) males did not show any component of sexual behavior, including simple mounting behavior (Ogawa et al, in press). This is similar to the ArKO mice and suggests that ERα and ERβ can complement one another in regard to this behavior.

In summary, through a more intensive study we have further characterized the fertility potential of male mice that lack the ability to synthesize estrogens. The ArKO mice sire significantly fewer litters than w/t mice do at age 14 weeks. This decrease in fertility may be attributable to spermatogenic disruptions, disruptions that appear to arise randomly with increasing age, possibly causing an impairment in the fertilization potential of mature spermatozoa. Conversely, the reduction in fertility could be attributable to a severe impairment in copulatory behavior. In conclusion, these results indicate that estrogen appears to play a crucial role in many areas of male reproduction, however, its exact function remains to be elucidated.

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References

Alexandre C, Balthazart J. Effects of metabolism inhibitors, antiestrogens and antiandrogens on the androgen and estrogen induced sexual behavior in Japanese quail. *Physiol Behav.* 1986;38:581–591.

Balthazart J, Evrard L, Surlemont C. Effects of the nonsteroidal inhibitor R76713 on testosterone-induced sexual behavior in the Japanese quail *(Coturnix coturnix japonica). Horm Behav.* 1990a;24:510–531.

Balthazart J, Foidart A, Harada N. Immunocytochemical localization of aromatase in the brain. *Brain Res.* 1990b;514:327–333.

Balthazart J, Surlemont C. Androgen and estrogen action in the preoptic area and activation of copulatory behavior in quail. *Physiol Behav.* 1990;48:599–609.

Beyer C, Wozniak A, Hutchison JB. Sex-specific aromatization of testosterone in mouse hypothalamic neurons. *Neuroendocrinology.* 1993; 58:673–681.

Carroll RS, Weaver CE, Baum MJ. Evidence implicating aromatization of testosterone in the regulation of male ferret sexual behavior. *Physiol Behav.* 1988;42:457–460.

Clancy AN, Zumpe D, Michael RP. Intracerebral infusion of an aromatase inhibitor, sexual behavior and brain estrogen receptor-like immunoreactivity in intact male rats. *Neuroendocrinology.* 1995;61:98–111.

Clemens LG, Pomerantz SM. Testosterone acts as a prohormone to stimulate male copulatory behavior in male deer mice *(Peromyscus maniculatus bairdi). J Comp Physiol Psychol.* 1982;96:114–122.

Cooper TT, Clancy AN, Karom M, Moore TO, Albers HE. Conversion of testosterone to estradiol may not be necessary for the expression of mating behavior in male Syrian hamsters *(Mesocricetus auratus). Horm Behav.* 2000;37:237–245.

D'Occhio MJ, Brooks DE. Effects of androgenic and oestrogenic hormones on mating behaviour in rams castrated before and after puberty. *J Endocrinol.* 1980;86:403–411.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology.* 1996;137:4796–4805.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab.* 1997;82:4258–4265.

Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA.* 1998;95:6965–6970.

Gonzalez MI, Leret ML. Role of monoamines in the male differentiation of the brain induced by androgen aromatization. *Pharmacol Biochem Behav.* 1992;41:733–737.

Graham-Lorence S, Amarneh B, White RE, Peterson JA, Simpson ER.

A three-dimensional model of aromatase cytochrome P450. *Protein Sci.* 1995;4:1065–1080.

Herbosa CG, Dahl GE, Evans NP, Pelt J, Wood RI, Foster DL. Sexual differentiation of the surge mode of gonadotropin secretion: prenatal androgens abolish the gonadotropin-releasing hormone surge in the sheep. *J Neuroendocrinol.* 1996;8:627–633.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. A role for oestrogens in the male reproductive system [see comments]. *Nature.* 1997;390:509–512.

Honda S, Harada N, Ito S, Takagi Y, Maeda S. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem Biophys Res Commun.* 1998;252:445–449.

Houtsmuller EJ, Brand T, de Jonge FH, Joosten RN, van de Poll NE, Slob AK. SDN-POA volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. *Physiol Behav.* 1994;56:535–541.

Hutchison JB. Gender-specific steroid metabolism in neural differentiation. *Cell Mol Neurobiol.* 1997;17:603–626.

Hutchison JB, Beyer C, Hutchison RE, Wozniak A. Sex differences in the regulation of embryonic brain aromatase. *J Steroid Biochem Mol Biol.* 1997;61:315–322.

Karolczak M, Kuppers E, Beyer C. Developmental expression and regulation of aromatase- and 5alpha-reductase type I mRNA in the male and female mouse hypothalamus. *J Neuroendocrinol.* 1998;10:267–274.

Krege JH, Hodgin JB, Couse JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci USA.* 1998;95:15677–15682.

Lacham-Kaplan O, Trounson A. The effects of the sperm motility activators 2-deoxyadenosine and pentoxifylline used for sperm microinjection on mouse and human embryo development. *Hum Reprod.* 1993;8:945–952.

Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J, Bunick D. Estrogen receptor alpha has a functional role in the mouse rete testis and efferent ductules. *Biol Reprod.* 2000;63:1873–1880.

Lephart ED, Simpson ER, McPhaul MJ, Kilgore MW, Wilson JD, Ojeda SR. Brain aromatase cytochrome P-450 messenger RNA levels and enzyme activity during prenatal and perinatal development in the rat. *Brain Res Mol Brain Res.* 1992;16:187–192.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA.* 1993;90:11162–11166.

Meisel R, Sachs B. The physiology of male sexual behavior. In: Knobil E, Neill J, eds. *The Physiology of Reproduction.* 2nd ed. New York, NY: Raven Press; 1994:3–106.

Negri-Cesi P, Poletti A, Celotti F. Metabolism of steroids in the brain: a new insight into the role of 5alpha-reductase and aromatase in brain differentiation and functions. *J Steroid Biochem Mol Biol.* 1996;58:455–466.

Nitta H, Bunick D, Hess RA, et al. Germ cells of the mouse testis express P450 aromatase. *Endocrinology.* 1993;132:1396–1401.

Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS, Pfaff DW. Survival of reproductive behaviors in estrogen receptor beta gene-deficient (betaERKO) male and female mice. *Proc Natl Acad Sci USA.* 1999;96:12887–12892.

Ogawa S, Lubahn DB, Korach KS, Pfaff DW. Behavioral effects of estrogen receptor gene disruption in male mice. *Proc Natl Acad Sci USA.* 1997;94:1476–1481.

Ogawa S, Washburn TF, Taylor J, Lubahn DB, Korach KS, Pfaff DW. Modifications of testosterone-dependent behaviors by estrogen receptor-alpha gene disruption in male mice. *Endocrinology.* 1998;139:5058–5069.

Parrott RF, Booth WD. Behavioural and morphological effects of 5 alpha-dihydrotestosterone and oestradiol-17 beta in the prepubertally castrated boar. *J Reprod Fertil.* 1984;71:453–461.

Robertson KM, O'Donnell L, Jones ME, et al. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci USA.* 1999;96:7986–7991.

Roselli CE, Resko JA. Aromatase activity in the rat brain: hormonal regulation and sex differences. *J Steroid Biochem Mol Biol.* 1993;44:499–508.

Roselli CE, Stormshak F, Resko JA. Distribution and regulation of aromatase activity in the ram hypothalamus and amygdala. *Brain Res.* 1998;811:105–110.

Santti R, Makela S, Strauss L, Korkman J, Kostian ML. Phytoestrogens: potential endocrine disruptors in males. *Toxicol Ind Health.* 1998;14:223–237.

Saunders PT, Fisher JS, Sharpe RM, Millar MR. Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. *J Endocrinol.* 1998;156:R13–R17.

Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? [See comments]. *Lancet.* 1993;341:1392–1395.

Simpson ER, Zhao Y, Agarwal VR, et al. Aromatase expression in health and disease. *Recent Prog Horm Res.* 1997;52:185–213.

Tobet SA, Baum MJ, Tang HB, Shim JH, Canick JA. Aromatase activity in the perinatal rat forebrain: effects of age, sex and intrauterine position. *Brain Res.* 1985;355:171–178.

van Pelt AM, de Rooij DG, van der BB, van der Saag PT, Gustafsson JA, Kuiper GG. Ontogeny of estrogen receptor-beta expression in rat testis. *Endocrinology.* 1999;140:478–483.

Wallis CJ, Luttge WG. Maintenance of male sexual behavior by combined treatment with oestrogen and dihydrotestosterone in CD-1 mice. *J Endocrinol.* 1975;66:257–262.

Watson JT, Adkins-Regan E. Testosterone implanted in the preoptic area of male Japanese quail must be aromatized to activate copulation. *Horm Behav.* 1989;23:432–447.

Wee BE, Weaver DR, Clemens LG. Hormonal restoration of masculine sexual behavior in long-term castrated B6D2F1 mice. *Physiol Behav.* 1988;42:77–82.

Wersinger SR, Rissman EF. Dopamine activates masculine sexual behavior independent of the estrogen receptor alpha. *J Neurosci.* 2000;20:4248–4254.

Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. *Horm Behav.* 1997;32:176–183.

Whitten PL, Naftolin F. Reproductive actions of phytoestrogens. *Baillieres Clin Endocrinol Metab.* 1998;12:667–690.